myconostica

# MycAssay™ Aspergillus

## Cepheid SmartCycler®
## Respiratory Samples
## REF 080-045

## Intended Use:

MycAssay™ Aspergillus is indicated for use by qualified laboratory professionals for the qualitative detection of *Aspergillus* spp. genomic DNA extracted from respiratory specimens from the lower respiratory tract (e.g., bronchial samples) as an aid to diagnosis in adult patients suspected of having *Aspergillus* infection or allergy.

MycAssay™ Aspergillus has been validated for use with the Cepheid SmartCycler® (using Dx software versions 1.7b and 3.0).

## Summary and Explanation

*Aspergillus* spp. are ubiquitous opportunistic moulds which cause both allergic and invasive syndromes. The genus is comprised of approximately 300 species, of which 41 have been associated with human disease. The majority of infections are caused by *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger;* less commonly, *A. nidulans* and other rarer species such as *A. sydowii, A. versicolor, A. lentulus and A. pseudofischeri* have been implicated[1]. Most infections and allergies caused by *Aspergillus* spp. affect the respiratory tract. Allergic syndromes include allergic bronchopulmonary aspergillosis (ABPA) and allergic *Aspergillus* sinusitis and are usually caused by *A. fumigatus*. Chronic pulmonary aspergillosis includes aspergilloma, chronic cavitary pulmonary aspergillosis and chronic fibrosing aspergillosis. Invasive aspergillosis (IA) occurs in at-risk patient groups including those treated with corticosteroids and those with

[1] Species Database in www.aspergillus.org.uk

neutropenia or phagocyte dysfunction (i.e. chronic granulomatous disease and HIV infection). About 80% of cases of invasive aspergillosis involve the lungs[2].

Routine diagnosis of invasive pulmonary aspergillosis includes Computed Tomography (CT) scanning, bronchoscopy and bronchoalveolar lavage (microscopy and culture), *Aspergillus* antigen testing of blood, or histological examination of surgical biopsy specimens. In this scenario, cultures are frequently falsely negative[3]. Indeed bronchoalveolar lavage is only positive by culture in approximately 40% of cases even when the diagnosis is proven by other means[4,5,6,7], showing the lack of sensitivity of culture in detecting invasive aspergillosis and chronic pulmonary aspergillosis. Cultures are, however, important if positive because many diagnostic tests do not indicate either the genus or species of fungus causing the disease, or the susceptibility profile of the isolate causing infection.

Allergic bronchopulmonary aspergillosis complicates asthma and cystic fibrosis[8] and may rarely present with no underlying disease. Most cases are associated with *A. fumigatus*, with other fungi occasionally implicated. Episodic airway obstruction with thick sputum plugs full of *Aspergillus* hyphae, serum total IgE >1,000 IU/mL and detectable *A. fumigatus* specific IgE and IgG antibodies or a positive *Aspergillus* skin prick test are the characteristics of the disease. Sputum cultures may be positive for *A. fumigatus* and bronchiectasis may be seen on a CT scan of the chest.

Current methods of diagnosing chronic pulmonary aspergillosis are a mixture of radiology (which is not specific)[9] and serology (*Aspergillus* IgG antibodies or precipitins) which is positive in most forms of aspergillosis (and thus not specific for any particular

[2] Hope WW, Walsh TJ, Denning DW. (2005). The invasive and saprophytic syndromes due to *Aspergillus* spp. Medical Mycology: 43 (suppl. 1): S207-38.
[3] Hope WW, Walsh TJ, Denning DW. (2005). Laboratory diagnosis of invasive aspergillosis. Lancet Infectious Diseases: 9: 609-22.
[4] Levy H, Horak DA, Tegtmeier BR, Yokota SB, Forman SJ. (1992). The value of bronchoalveolar lavage and bronchial washings in the diagnosis of invasive pulmonary aspergillosis. Respir Med: 86: 243-8.
[5] Greub G and Bille J. (1998) Aspergillus species isolated from clinical specimens: suggested clinical and microbiological criteria to deteremine significance. Clin Microbiol Infect 4: 710-716.
[6] Perfect JR, Cox GM, Lee JY, Kauffman CA, de Repentigny L,Chapman SW, Morrison VA, Pappas P, Hiemenz JW, Stevens DA, and the Mycoses Study Group. (2001). The impact of culture isolation of *Aspergillus* species: A hospital-based survey of Aspergillosis. Clinical Infectious Diseases; 33:1824–33.
[7] Maertens J, Van Eldere J, Verhaegen J, Verbeken E, Verschakelen J, Boogaerts M. (2002). Use of Circulating Galactomannan Screening for Early Diagnosis of Invasive Aspergillosis in Allogeneic Stem Cell Transplant Recipients The Journal of Infectious Diseases. 186:1297–306.
[8] Tillie-Leblond I, Tonnel AB. (2005). Allergic bronchopulmonary aspergillosis. Allergy: 60: 1004-13.
[9] Greene R. (2005). The radiological spectrum of pulmonary aspergillosis. Med Mycol: 43 (Suppl 1): S147-54.

manifestation of aspergillosis)[1]. Cultures are positive in only 40-65% of cases proven by radiology and serology[10,11]. As the differential diagnosis is wide including tuberculosis, atypical mycobacteriosis, sarcoidosis, histoplasmosis, coccidioidomycosis, pneumoconiosis, rheumatoid lung, ankylosing spondylitis and others, documenting the presence of *Aspergillus* spp. in respiratory samples is important to prevent delay in the recognition of pulmonary aspergillosis and mistreatment .

MycAssay™ Aspergillus is a molecular diagnostic kit for the detection of *Aspergillus* spp. genomic DNA using Molecular Beacon[12] Real-Time PCR technology. The whole test procedure, including extraction of DNA from the clinical sample, can be completed within 4 hours, compared to fungal culture which can take several days to produce positive results. This assay offers advantages over currently available diagnostic methods for acute invasive and chronic pulmonary aspergillosis. These advantages include faster detection of *Aspergillus* spp. and the potential for increased sensitivity for *Aspergillus* spp. in highly immunocompromised patients suspected of having invasive pulmonary aspergillosis.

## Principles of the Procedure

Following mixing of the reagents in the MycAssay™ Aspergillus kit with a sample containing the *Aspergillus* target DNA sequence (a section of the *Aspergillus* ribosomal 18S gene), thermocycling will result in DNA amplification occurring. The assay also contains an Internal Amplification Control (IAC), a DNA fragment not present in Aspergilli, other fungal, bacterial or human genomes, to detect PCR inhibitory substances and confirm the functionality of the assay reagents.

The amplified DNA targets are detected using Molecular Beacon technology. Molecular Beacons are single-stranded oligonucleotide hybridisation probes that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore, which fluoresces

[10] Denning DW, Riniotis K, Dobrashian R, Sambatakou H. (2003). Chronic cavitary and fibrosing pulmonary and pleural aspergillosis: Case series, proposed nomenclature and review. Clin Infect Dis: 37 (Suppl 3): S265-80.
[11] Camuset J, Lavolé A, Wislez M, Khalil A, Bellocq A, Bazelly B, Mayaud C, Cadranel J. (2007). Bronchopulmonary aspergillosis infections in the non-immunocompromised patient. Rev Pneumol Clin. 63:155-166.

[12] Tyagi S, Kramer FR. (1996). Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology: 14: 303-308.

when excited by light of the appropriate wavelength, is covalently linked to the end of one arm and a quencher, which suppresses the fluorescence of the fluorophore when in close physical proximity, is covalently linked to the end of the other arm. Molecular beacons do not fluoresce when they are free in solution. However, when they hybridise to a nucleic acid strand containing a target sequence they undergo a conformational change that physically separates the fluorophore and the quencher enabling them to fluoresce upon excitation. The amount of fluorescence at any given cycle, or following cycling, depends on the amount of specific amplicons present at that time. The Real-Time PCR System simultaneously monitors the fluorescence emitted by each beacon.

## Precautions

- The kit is intended for use only by laboratory professionals. Procedures are required for non-aerosol manipulations of specimens. Standard precautions and institutional guidelines should be followed in handling all samples. A Material Safety Data Sheet is available from Myconostica Ltd.
- This test is for *in vitro* diagnostic use only.
- This test is only for use with the Cepheid SmartCycler® system with Dx diagnostic software versions 1.7b and 3.0
- Do not use reagents or controls if the protective pouches are open or broken when received.
- Reagents and controls are not interchangeable between kits with different lot numbers.
- Never pool reagents or controls from different tubes even if they are from the same lot.
- Never use the reagents or controls after their expiry date.
- Reagents and controls should not be re-frozen or re-used after opening.
- Wear protective clothing and disposable gloves while handling kit reagents.
- Avoid microbial and deoxyribonuclease (DNAse) contamination of reagents when removing aliquots from tubes.
- The use of sterile, DNAse-free, low-retention disposable filter-tips or positive displacement pipette tips is recommended.
- Use a new tip for each specimen or reagent.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- To avoid contamination with *Aspergillus* or IAC amplicons, do not open the reaction tubes after amplification.

- Additional controls may be tested according to guidelines or regulations of local, state, provincial, federal or accrediting organisations.
- Do not eat, drink or smoke in areas where specimens or kit reagents are being handled.
- Low concentrations of DNA can be unstable if not stored correctly. It is recommended that DNA extractions from clinical samples are stored at -80°C to preserve their integrity. Multiple rounds of thawing and refreezing should also be avoided whenever possible.

## Kit Contents

Description

The kit consists of five 3-compartment sealed foil pouches each of which can be removed from the box and used separately. Each pouch contains sufficient reagents for 8 reactions.

| | | Volume |
|---|---|---|
| **Tube 1** (Orange Cap) | dNTPs<br>$MgCl_2$<br>Buffered solution of DNA Polymerase complex | 66 µL |
| **Tube 2** (Green Cap) | <0.01% Primers<br><0.01% Molecular Beacons<br><0.0001% Internal Amplification Control (IAC)<br>The Internal Amplification Control is a recombinant DNA plasmid containing a non-infective sequence unrelated to target (*Aspergillus*) sequence<br>Tris-HCl Buffer | 66 µL |
| **Tube 3** (Clear Cap) | Negative Control<br>Water | 25 µL |
| **Tube 4** (Black Cap) | Positive Control<br><0.0001% Positive Control DNA<br>The Positive Control molecule is a recombinant plasmid containing the *Aspergillus* target sequence | 25 µL |

Tris-HCl Buffer

The kit also contains:

- MycAssay™ Aspergillus Myconostica Protocol CD-ROM
- Instructions for Use
- Certificate of Analysis

## Storage

The kit should be stored frozen (-15 to -25 °C) until the expiry date indicated on the kit box label, when it should be disposed of according to local regulations.

Once a pouch has been opened, the contents must be used immediately, not re-frozen or re-used at a later date.

## Equipment/Materials required but not provided

A. Equipment required
- SmartCycler® Real-Time PCR System (including user manual, attached computer and SmartCycler® Dx software versions 3.0 or 1.7b)
- SmartCycler® reaction tubes
- Mini centrifuge adapted for SmartCycler® reaction tubes
- Plastic support rack for SmartCycler® reaction tubes

B. Common equipment required
- Micro centrifuge
- Vortex mixer
- Micropipettes (volumes required 7.5 µL – 20 µL)
- Sterile low-retention filtertips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- Permanent marker pen
- DNA isolation kit (see below)

## Specimen

The specimen for the MycAssay™ Aspergillus assay is total genomic DNA extracted from clinical BAL and other lower respiratory tract samples. The following isolation kit and equipment is recommended for this purpose and was used during validation:

- MycXtra™ Fungal DNA Extraction kit (REF: 080-005 available from Myconostica)
- Vortex-Genie 2 (Scientific Industries Inc., New York, USA)
- Vortex Adaptor Plate (REF: 080-015 available from Myconostica)

## Procedural Notes

- Read the entire protocol before commencing.
- The entire MycAssay™ Aspergillus process (excluding DNA extraction) takes approximately 2 hours, dependent on the number of samples tested.
- Setting up of the test should be performed in a PCR workstation or pre-PCR laboratory. If a PCR workstation is not available, then the test should be set-up in a dedicated area of the laboratory[13], separated from areas used for DNA extractions, that is regularly cleaned with DNA decontaminating reagents.
- However, avoid using DNA decontaminating reagents when performing the Real-Time PCR set-up as they can inhibit the assay.
- Use micropipettes for the transfer of fluids. Dedicated micropipettes should be used for the set-up of these reactions and they should be regularly decontaminated.
- Low-retention filtertips are recommended for use to ensure that no DNA is lost during the set-up procedure.
- **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**
- Wear gloves at all times.
- All reagent tubes must be capped following use and prior to disposal.
- Take care to identify the SmartCycler® reaction tubes appropriately when multiple patient samples are being processed.
- Take care when selecting the protocol run file: Select ONLY **MycAssay Aspergillus Dx1,7b v1.3** or **MycAssay Aspergillus v1_3**; do NOT select **SERUM MycAssay Asp v1**.

[13] For example see Mifflin, T. E. (2003). Setting up a PCR Laboratory. *In* PCR Primer, 2nd Ed. (eds. Dieffenbach and Dveksler). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. USA.

## Procedure for Use:

### 1. Real-Time PCR Set-Up

1.1 To begin, switch on the Real-Time PCR System (instrument and associated computer) and launch the relevant software. Enter usernames and passwords as required.

1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.

1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 patient samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. A maximum of 38 patient samples may be tested using the 5 pouches in a kit.

1.4 Calculate the number of reactions required, referring to the table below:

| Number of Pouches | Maximum number of patient samples |
|---|---|
| 1 | 6 |
| 2 | 14 |
| 3 | 22 |
| 4 | 30 |
| 5 | 38 |

1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the patient samples were frozen after extraction, also remove these from the freezer.

1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**

1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed before proceeding. Vortex mix the tubes' contents and the patient samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use.

1.8 Place the required number of SmartCycler® reaction tubes in their support rack(s). Take care to only touch the neck of the reaction tubes with your hands.

1.9 Always set up the negative control first, followed by the patient samples. The positive control should always be set up last.

1.10 Reagent and DNA volumes are shown in the table below:

| Reagent | Reaction | | |
|---|---|---|---|
| | **Negative control** | **Patient sample** | **Positive control** |
| **Tube 1 (Orange cap)** | 7.5 µL | 7.5 µL | 7.5 µL |
| **Tube 2 (Green cap)** | 7.5 µL | 7.5 µL | 7.5 µL |
| **Tube 3 (Clear cap)** | 10 µL | - | - |
| **Patient Sample** | - | 10 µL | - |
| **Tube 4 (Black cap)** | - | - | 10 µL |
| Total volume | 25 µL | 25 µL | 25 µL |

1.11 Add reagents in the order shown in the table above; Tube 1, then Tube 2, followed by the template (Negative control, Patient sample, or Positive control). Take care when taking aliquots from Tube 1; the liquid is slightly viscous and can stick on the inner ridge of the tube. If this happens, re-spin to collect the final contents in the base of the tube before attempting to remove the final aliquots.

1.12 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.

1.13 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction tube. Closing the lids on the other

reaction tubes before opening Tube 4 can reduce the risk of cross-contamination.

1.14 Make sure all reaction tube lids are firmly closed and then label each lid using a permanent marker pen e.g. POS for positive control, NEG for negative control and patient ID for patient samples. Spin down the reaction tubes for 10 seconds using the specially-adapted mini centrifuge. Visually check that there are no bubbles present in the reaction mixtures.

1.15 Proceed to Section 2 promptly. MycAssay™ Aspergillus reactions are stable on the bench for up to 60 minutes.

1.16 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

## 2. Performing the run

Before proceeding with the following section, please check which version of the Dx software you have installed on your computer. Open the software, choose **Help** from the toolbar and click **About**.

For version 1.7b, follow the instructions below in Section 2.1
For version 3.0, follow the instructions below in Section 2.2

Please also be aware that certain user privileges are required in the software to **Retrieve Run(s)** or **Import** an assay. These can only be assigned by the **Administrator** of the instrument.

## 2.1 SmartCycler® Dx Diagnostic software version 1.7b

2.1.1 Open up the SmartCycler® Dx Diagnostic software version 1.7b and enter your username and password.

2.1.2 Insert the **MycAssay Aspergillus Myconostica Protocol CD-ROM** and click on the **Define Assays** tab.

2.1.3 Got to **Retrieve Run(s)** via the **Tools** directory on the top menu bar and click **Proceed**:

2.1.4 Select the file **MycAssay Aspergillus Dx1_7bv1_3.DXA** from the CD-ROM as shown below. This file should be one of two recognised by the software.

2.1.5 On the next screen highlight the filename **MycAssay Aspergillus Dx1,7b v1.3** and click **OK**, followed by **Proceed** and **OK**.

2.1.6 Close the software. When it is reopened the **MycAssay Aspergillus Dx1.7b v1.3** assay will be available for use when creating a new run.

2.1.7 Click on the **Create Run** tab. Enter an appropriate **Run Name** (it is recommended that this includes the date and operators initials as a minimum), or leave blank if you wish the name to be created automatically by the software.

2.1.8 Select **MycAssay Aspergillus Dx1.7b v1.3** as the assay.

2.1.9 Enter the **Lot Number** and **Expiration Date** of the kit as printed on the kit box and on each pouch. The lot number will be in the form of M-XXXXXXXX.

2.1.10 Enter the **Number of specimens** in the box and click **Apply**. The **Sample ID** for each specimen will automatically be named *SPEC* by the software. Therefore, rename each site appropriately for identification purposes; i.e. double click on *SPEC* to highlight it and then type in the sample ID.

The software will automatically include a Negative and Positive control in the Real-Time PCR run.

2.1.11 Carefully place the reaction tubes into the designated sites in the SmartCycler® block and click **Start Run**. N.B. Take care when placing the reaction tubes into the designated sites as they may not be in the same order as your set-up. Make a note of the run name and click **OK**. The run will now start and red lights will appear above each site in use on the block.

2.1.12 To determine how long the run will take to complete, click on the **Check Status** tab. The run name and subsequent run time will be listed.

## 2.2 SmartCycler® Dx Diagnostic software version 3.0

2.2.1 Open up the SmartCycler® Dx Diagnostic software version 3.0 and enter your username and password.

2.2.2 Insert the **MycAssay Aspergillus Myconostica Protocol CD-ROM** and click on the **Define Assays** tab, and **Import** the **MycAssay Aspergillus v1_3.sca** file from the CD-ROM.

2.2.3 Click on the **Create Run** tab. Enter an appropriate **Run Name** (it is recommended that this includes the date and operators initials as a minimum), or leave blank if you wish the name to be created automatically by the software.

2.2.4 Select **MycAssay Aspergillus v1.3** as the assay.

2.2.5 Enter the **Lot Number** and **Expiration Date** of the kit as printed on the kit box and each pouch. The lot number will be in the form of M-XXXXXXXX.

2.2.6 Enter the **Patient (Sample) ID** and the **Number of specimens** (replicates) in the appropriate boxes and click **Apply**. Do this for all patient samples being tested. The software will automatically include a Negative and Positive control in the Real-Time PCR run.

2.2.7 Carefully place the reaction tubes into the designated sites in the SmartCycler® block and click **Start Run**. N.B. Take care when placing the reaction tubes into the designated sites as they may not be in the same order as your set-up. Make a note of the run name and click **OK**. The run will now start and red lights will appear above each site in use on the block.

2.2.8 To determine how long the run will take to complete, click on the **Check Status** tab. The run name and subsequent run time will be listed.

## 3. Data Analysis and Interpretation

3.1 The results can be viewed in Dx software, by selecting the **View Results** tab.

3.2 Click on the **View Another Run** button at the bottom of the page, select the run you wish to view then click **OK**.

3.3 The **Patient Results** should already be selected in the **Views** list. The patient (sample) ID and the subsequent assay result will be clearly listed. The results can be interpreted using the table below:

| Out-come | Patient Result | Colour | Interpretation | Further Action |
|---|---|---|---|---|
| 1 | Negative | Green | Negative for *Aspergillus* | Report result |
| 2 | Positive | Red | Positive for *Aspergillus* | Report result |
| 3 | Invalid | Light Grey* | IAC failure in sample | Repeat sample |
| 4 | Invalid | Light Grey* | Failure in Positive or Negative Control | Repeat entire run |

*If the result is reported as ND, in light grey, this corresponds to error code 3079, the result of high fluorescence in one or more channels. If a Ct value of ≤36.0 is recorded in the *Aspergillus* channel, report as positive.

3.4 To view individual sample results for either *Aspergillus* or IAC separately, select **Sample Results** from the **Views** list and click on the individual tabs for each target. The results will be displayed in the same format as the **Patient Results** but for each individual target.

3.5 If a Patient sample reports an Invalid result, this is due to a failed IAC result (indicated by Unresolved in the **Sample Results** tab); repeat the reaction (plus Positive and Negative controls). If the reaction continues to fail, an inhibiting substance may be present in the template and a Negative result cannot be relied upon.

3.6 To export run data to allow transfer to another computer, go to the **Tools** directory at the top of the screen and select **Data Management**, followed by **Archive Run(s)** from the drop down menu. A message screen will appear, click **Proceed.** Select the run to be archived by ticking the box to the left and click **OK**. A warning message will appear stating how many runs are to be

archived; if this number is correct, click **Proceed**. Select a destination to save the run file e.g. USB data stick. Click **Save** and make a note of the file name. A message screen will appear stating how many runs are to be archived, if this number is correct, click **Proceed.**

3.7 To import run data, go to the **Tools** directory at the top of the screen and select **Data Management**, followed by **Retrieve Run(s)** from the drop down menu. A message screen will appear, click **Proceed.** Go to **Look In:** and select the storage device used to archive the run data (see section 3.4 above). Select the run file to be retrieved and click **Open**. Another screen will appear prompting you to select the run you wish to retrieve. Select the run to be retrieved and click **OK**. A message screen will appear stating how many runs are to be retrieved, if this number is correct, click **Proceed.**

3.8 If a hardcopy of the results is also required, click on **Report** and **Print**.

## 4. Troubleshooting

### 4.1 The Negative Control has generated a positive signal in the FAM channel:

- Contamination occurred during the set up. Results from the entire run cannot be relied upon as accurate.

→ Repeat the entire run taking great care when adding the templates, in particular, the Positive Control (Tube 4), to ensure that cross-contamination does not occur.

→ Make sure that the work area and instruments are properly decontaminated before and after use.

- The Negative Control was incorrectly positioned in the instrument.

→ Take care that the reaction tubes are placed in their designated sites.

### 4.2 The Negative Control IAC Ct value is not within the acceptable range:

- The PCR has been inhibited.

→ Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with unexpired kit if necessary.

- Either Tube 1 or 2 reagent was not added to the PCR reaction, or double the amount of Tube 2 was added.

→ Repeat the run taking care in the set-up stage. Such errors can be detected by seeing higher or lower levels of liquid in one reaction tube compared to others.

**4.3 The Positive Control is negative:**

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.

- An error occurred during step 1.12 and the Positive Control template (Tube 4) was placed in the wrong reaction tube.

→ Repeat the run, taking great care during the set-up stage. Such errors can be detected by seeing a higher level of liquid in one reaction, and a lower level in another, compared to normal.

- Either Tube 1 or 2 reagent was not added to the reaction.

→ Repeat the run taking care in the set-up stage. Such errors can be detected by seeing lower levels of liquid in this reaction compared to others.

- The Positive Control was incorrectly positioned in the instrument.

→ Take care that the reaction tubes are placed in their designated sites.

**4.4 Patient sample(s) give Outcome 3 - "Invalid":**

- It is likely that the extracted clinical sample(s) contain PCR inhibitors.

→ We recommend that DNA from clinical samples is extracted using the MycXtra™ Fungal DNA Extraction kit.

**4.5 There are no results for any channel with any samples or controls:**

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.

- The equipment used is not functioning optimally.

→ Please check that your Real-Time PCR instrument has an up-to-date service history and has been fully calibrated as described in its Installation and Maintenance Guide.

- An incorrect protocol file was used during the software set up.

→ Please refer to Section 2 and choose the correct Protocol file, as specified for each software type/version, from the Myconostica Protocol CD-ROM. Only the file appropriate to the software can be loaded. Repeat the run using the correct protocol file.

If you have further questions, or you experience any problems, please contact Technical Support (productsupport@lab21.com)

# Performance Characteristics and Limitations

## Analytical Sensitivity

Using the protocol described above, and PCR templates generated at Myconostica, the Limit of Blank (LoB) for the MycAssay™ Aspergillus was determined to be a Ct of 38.0, while the Limit of Detection (LoD) was determined to be <50 copies of target DNA. This was determined using the AF293 strain of *Aspergillus fumigatus* from which the genome has been fully sequenced. It is known there are 37 copies of the target within the genome, determined by optical mapping[14] and thus 50 target copies represents approximately 1.3 genomes.

## Analytical Specificity and Selectivity

Analytical specificity was tested using DNA extracted from 15 different Aspergilli species, including several strains each of *A. fumigatus, A. niger, A. terreus,* and *A. nidulans*. Signals detected above the LoB were recorded as a positive result.

All of the 15 *Aspergillus* spp. tested were positive with the assay. In addition to those previously mentioned, this includes *A. flavus, A. versicolor, A. glaucus, A. sclerotiorum, A. niveus, A. lentulus, A. unguis, A. candidus, A. wentii, A. tubingensis and A. foetidus*.

Genomic DNA extracted from *Penicillium* spp. also generated positive results. This is due to the fact that the sequences of the molecular targets are highly conserved between *Aspergillus* and *Penicillium*. Therefore, it must be noted that a positive result with this assay may be the result of infection by *Penicillium*, rather than *Aspergillus*.

Analytical selectivity was tested using DNA extracted from a variety of different fungal and non-fungal species. The following species did not report out a positive result;
*Alternaria alternata Blastomyces capitatus, Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, Cladosporium spp., Cryptococcus neoformans, Doratomyces microsporus, Fusarium solani, Histoplasma capsulatum Pneumocystis jirovecii, Rhizomucor pusillus, Rhodotonila rubra, Saccharomyces cerevisiae, Scedosporium*

[14] Nierman WC, Pain A, Anderson MJ, et al. (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigates.* Nature: 438: 1151-6.

*apiosperinu, S. prolificans, Sporothrix schenkii, Trichosporon capitatu*. The following bacterial species did not report a positive result; *Bordetella pertussi, Corynebacterium diphtheriae, Escherichia coli, Haemophilus influenza, Lactobacillus plantarum, Legionella pneumophila Moraxella catarrhalis, Mycoplasma pneumonia, Neisseria meningitides, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumonia, S. pyogenes, S. salivarius*.

Human genomic DNA does not report a positive result with this assay.

### Reproducibility and Repeatability

Repeatability and reproducibility were determined by 5 different operators testing a blind panel of 7 different templates, in triplicate. Experiments were performed using 3 different manufactured batches of MycAssay™ Aspergillus kit, on 3 different instruments situated at 2 different locations in the UK.

The results were analysed against the Limit of Blank (LoB) and the clinical cut-off (CCO). At a concentration of 3 times the Limit of Detection (LoD), the results from 100% of all samples tested were in agreement (positive) for LoB, and 91% of the samples were in agreement at the CCO. At concentrations higher than 3 x LoD, results from 100% of all samples were in agreement at both the CCO and LoB. For negative templates, all samples tested were negative at the CCO.

### Interfering Substances (contraindications for use)

The following compounds were tested at clinically relevant concentrations, and found not to inhibit the assay; acteylcysteine, amphotericin, beclometasone dipropionate, budesonide, colistimethate sodium, fluticasone propionate, formoterol fumarate dehydrate, ipratropium bromide, lidocaine, mannitol, salbutamol sulphate, salmerterol, sodium chloride, sodium cromoglicate, terbutaline, tobramycin.

## Performance Evaluation

Respiratory samples (BAL) that had been collected from 2 hospitals, extracted using the MycXtra™ kit, and stored were used to evaluate the performance of the MycAssay™ Aspergillus kit with clinical samples. Comparisons were made to both clinical diagnosis and culture.

The cut-off value of a Ct of 36.0 was established following review of a dataset of samples sourced from different sites and different patient populations. Different cut-offs were evaluated for the probability of differentiating between disease state and non-disease state.

**PCR v Clinical Diagnosis**

| | Clinical positive | Clinical negative | | |
|---|---|---|---|---|
| PCR positive | 31 | 1 | 0.97 | PPV |
| PCR negative | 2 | 10 | 0.83 | NPV |
| | 0.94 | 0.91 | | |
| | Sensitivity | Specificity | | |

**PCR v *Aspergillus* Culture**

| | Culture positive | Culture negative | | |
|---|---|---|---|---|
| PCR positive | 29 | 3 | 0.91 | PPV |
| PCR negative | 2 | 10 | 0.83 | NPV |
| | 0.94 | 0.77 | | |
| | Sensitivity | Specificity | | |

Of the samples tested, 0.8% contained PCR inhibitors as reported by the IAC, following extraction using the MycXtra™ kit.

**Clinical Reporting**

NOTE: When inspecting the Results Report ensure that the correct protocol has been used. For respiratory samples MycAssay Aspergillus Dx1,7b v1.3 or MycAssay Aspergillus v1_3 must be used. The SERUM MycAssay Asp v1 protocol must only be used with the MycAssay Aspergillus SmartCycler Serum IFU (Part No. 030-169). Use of the wrong protocol will give invalid results.

The MycAssay™ Aspergillus kit is intended as an aid to diagnosis. The results need to be taken in context of the clinical condition of the patient and other diagnostic test results.

The following are recommended reports, each depending on the assay result interpretation:

Outcome No 1
"*Aspergillus* spp. not detected"

Outcome No 2
"*Aspergillus* spp. detected; Positive result. This assay also detects *Penicillium* spp."

Outcome No 3
"Test failed; inhibitors or other unknown substance present"

## Limitations of Procedure

- The principal limitation of this procedure relates to the quality of the primary sample:
  - If the sample is very small or not collected from the affected area of lung, the test will be less sensitive and may be falsely negative.
  - BAL samples should be centrifuged prior to DNA extraction from the pellet.
  - Data also demonstrated that a reduction in the volume of supernatant used in the extraction process, achieved by the centrifugation step, decreases the proportion of inhibitors entering the system.

- False positive results are possible if the infecting agent is *Penicillium* spp. which cannot be differentiated from *Aspergillus* spp. using this kit.

- While the MycXtra™ Fungal DNA extraction procedure is designed to remove PCR inhibitors, not all drugs or patient populations have been evaluated.

- The procedure has not been fully assessed with sputa nor has it been assessed with induced saline samples or on samples from children.

- False positive results may arise from external contamination of the original sample or test. Such contamination could arise from *Aspergillus* contaminated air, poor experimental technique with respect to the positive control or external (especially pipettor) contamination with *Aspergillus* DNA.

- As a true positive result may be obtained from patients who are transiently or persistently colonised by *Aspergillus* spp., clinical judgment is required in interpretation of the test results, in the context of disease.

## LICENSING

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